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Influence of Shifting Positions of Ser, Thr, and Cys Residues in Prenisin on the Efficiency of Modification Reactions and on the Antimicrobial Activities of the Modified Prepeptides▽†

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Since the recent discovery that the nisin modification and transport machinery can be used to produce and modify peptides unrelated to nisin, specific questions arose concerning the specificity of the modification enzymes involved and the limits of their promiscuity with respect to the dehydration and cyclization processes. The nisin leader peptide has been postulated to fulfill a recognition and binding function required for these modifications. Here, we investigated whether the relative positions of the modifiable residues in the nisin prepeptide, with respect to the leader peptide, could influence the efficiency of their modification. We conducted a systematic study on the insertion of one to four alanines in front of either ring A or ring D to change the "reading frame" of modifiable residues, resulting in altered distance and topology of the modifiable residues relative to the leader. The insertion of N-terminal and hinge-located Ala residues had only a modest influence on the modification efficiency, demonstrating that the "phasing" of these residues relative to the leader peptide is not a critical factor in determining modification. However, in all cases, but especially with the N-terminal insertions, the antimicrobial activities of the fully modified nisin species were decreased.

Lantibiotics are antimicrobial peptides produced by gram-positive bacteria that contain unusual dehydrated amino acids and lanthionine rings. Nisin, produced by *Lactococcus lactis*, is one of the first known and best studied lantibiotics (15, 22, 27). Nisin acts against many gram-positive species, including food spoilage bacteria (e.g., *Bacillus cereus* and *Listeria monocytogenes*), and because of its potent antimicrobial activity and natural origin, nisin is broadly used as a food preservative (2). It is synthesized ribosomally in the form of a prepeptide which contains 57 amino acid residues. The unmodified nisin molecule contains a leader sequence comprising 23 amino acid residues, which are cleaved off at the end of the maturation process, and a structural part, which undergoes posttranslational modifications to become mature nisin. The leader sequence of nisin is presumed to be necessary for the targeting and binding of the nisin prepeptide to the transport and modification machinery (9, 10, 12, 13, 24). During the maturation process, specific serines and threonines within the nisin peptide are modified by enzymes called NisB and NisC (10), which, together with the integral membrane protein NisT, are believed to form a membrane-associated synthetase complex (24). NisB dehydrates serines and threonines into dehydroalanines and dehydrobutyrines, respectively (7, 9). Dehydrated residues are coupled to cysteines by NisC to generate unique structures called (methyl)lanthionine rings (7, 13). Modified nisin is transported across the lipid bilayer by the dedicated ABC transporter NisT (8, 10, 16). Subsequently, in order to liberate active nisin, its leader peptide is extracellularly cleaved off by a protease called NisP (10, 25).

Genes responsible for nisin biosynthesis and immunity are organized in a gene cluster and are located on a conjugative transposon, Tn*5276* (3, 10, 17). Until recently, it was believed that the nisin biosynthetic enzymes form an orchestrated set of proteins with narrow substrate specificity. NisBTC proteins were demonstrated to interact with each other by coimmunoprecipitation studies and yeast two-hybrid screens, leading to the proposal of a multimeric membrane-associated synthetase complex. However, recent studies indicate that these three enzymes can also be active independently (8, 9). Moreover, in contrast to what has been assumed before, the enzymes involved in nisin modification and transport were shown to display a broad substrate specificity (6, 8, 9, 18, 21). The nisin biosynthetic machinery has been demonstrated to modify and transport peptides which are totally unrelated to nisin, provided that they are fused to the nisin leader peptide (6). These findings raised an opportunity to use the nisin biosynthetic enzymes as a tool to introduce (methyl)lanthionine rings into biotechnologically and pharmaceutically valuable peptides. (Methyl)lanthionine structures are rather uncommon in nature and can modulate the activity and increase the proteolytic stability of peptides (6).

The discovery that the activity of the dehydratase NisB is not restricted only to the nisin template, and that other peptides can also serve as a substrate for this enzyme, raised a number of questions. What determines the substrate specificity of NisB? Is NisB capable of dehydrating serines and threonines in any sequence when fused to the nisin leader, and does the distance between the serines and threonines and the leader matter? Even though it was demonstrated that nonlantibiotic peptides can be successfully dehydrated by NisB (6), some

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TABLE 1. *Lactococcus lactis* strain and plasmids used in the study

Strain or plasmid	Characteristic(s)	Reference
Strain		
NZ9000	*nisRK*	5
Plasmids		
pIL3BTC	*nisBTC*, encoding nisin modification machinery	19
pNZnisA-E3	*nisA*, encoding nisin	8
pNZE3-nis-Ala1	*nisA*, encoding nisin, with one alanine inserted after position 1	This study
pNZE3-nis-Ala2	*nisA*, encoding nisin, with two alanines inserted after position 1	This study
pNZE3-nis-Ala3	*nisA*, encoding nisin, with three alanines inserted after position 1	This study
pNZE3-nis-Ala4	*nisA*, encoding nisin, with four alanines inserted after position 1	This study
PNZE3-nis-HAla1	*nisA*, encoding nisin, with one alanine inserted after so-called hinge region, position 22	This study
PNZE3-nis-HAla2	*nisA*, encoding nisin, with two alanines inserted after so-called hinge region, position 22	This study
PNZE3-nis-HAla3	*nisA*, encoding nisin, with three alanines inserted after so-called hinge region, position 22	This study
PNZE3-nis-HAla4	*nisA*, encoding nisin, with four alanines inserted after so-called hinge region, position 22	This study

serines and threonines can escape NisB-mediated dehydration. Nisin contains three dehydrated amino acids and five thioether rings. Serines and threonines in the leader peptide, which is cleaved off by NisP, as well as serine at position 29 in nisin, are never dehydrated. Furthermore, serine at position 33 sometimes escapes dehydration. What is the reason that these particular residues are not dehydrated? The leader sequence is postulated to be a binding domain of prenisin which interacts with nisin transport and modification enzymes. Thereby, serines and threonines within the amino acid sequence of the leader are protected against dehydration, and the dehydration reaction is possible only upon and after the binding of the leader peptide to the NisB enzyme. Rink and colleagues analyzed primary sequences of various lantibiotics in order to investigate the substrate specificity of NisB (19, 21). This led to the conclusion that serines and threonines which are dehydrated are flanked more often by hydrophobic residues than by hydrophilic ones (19, 21). These in silico findings were supported experimentally by the analysis of the dehydration pattern of artificial hexapeptide fusions with the leader sequence of nisin. Hydrophobic residues that directly flank serines and threonines favored dehydration, whereas hydrophilic amino acids flanking serines and threonines on both sides and especially negatively charged amino acids disfavored dehydration. In this way, guidelines for the design of modifiable peptides were obtained, but no absolute rules have been established (19, 21). These guidelines implied that the flanking amino acids of modifiable residues play a role in determining the substrate specificity of NisB.

Here, we have investigated whether the "reading frame" or "phasing" of nisin A is also important for the dehydration and cyclization reaction. The position of serines and/or threonines relative to the leader peptide, which has been postulated to dock the substrate onto NisB and NisC, might have an important implication for their processing. Many nisin mutants have been generated, but the lanthionine ring pattern was usually unaltered in these mutants (20). Here, we have generated nisin mutants with shifted dehydratable residues and ring positions for the first time. Although it has been demonstrated before that peptides other than nisin can be processed by NisB, the distance between the nisin leader peptide and the dehydratable residues was either identical or very similar in those peptides to the distance between wild-type nisin and the dehydratable residues (6, 19). In order to investigate the importance of the positioning of serines or threonines in nisin-related peptides relative to the leader for their dehydration, we have created a series of alanine insertion mutants. In these mutants, the distance of modifiable serines, threonines, and cysteines relative to the leader peptide was systematically enlarged by one to four amino acids. We show that the nisin-dehydrating enzyme NisB displays a relaxed substrate specificity for the resulting nisin-derived substrate peptides and that the relative distance between the leader peptide and dehydratable residues is not a main determinant for the dehydration reaction to occur. However, antimicrobial activity analysis of the eight mutants showed a strong negative influence of N-terminal additions of alanines, whereas the same additions in the flexible "hinge region" of nisin allow the successful generation of new peptide antibiotics with significant activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids which were used in this study are listed in Table 1. We have used a double-plasmid expression system as described before (6). Plasmid pIL3BTC was used to produce nisin modification machinery, and a second plasmid, pNZnisA-E3, coded for the nisin A peptide. The latter plasmid was mutagenized in order to obtain various nisin mutants. The NZ9000 strain was used as a host for the nisin-inducible expression of *nisBTC* genes in conjunction with the production of nisin and its mutants (11).

Cells were grown at 30°C in M17 medium (Difco) and supplemented with 0.5% (wt/vol) glucose, and the following antibiotics were used when appropriate: chloramphenicol, 5 μg/ml, and erythromycin, 2 μg/ml.

Mass spectrometry analysis. Cultures of producer strains grown overnight were prepared using C_{18} ZipTips (Millipore) for a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis essentially as described before (8). Briefly, ZipTips were equilibrated with 100% acetonitrile and washed with 0.1% trifluoroacetic acid. Subsequently, the supernatant containing peptides was mixed with 0.1% trifluoroacetic acid and applied to a ZipTip. Bound peptides were washed with 0.2% trifluoroacetic acid and eluted with 50% acetonitrile and 0.1% trifluoroacetic acid. The eluent was mixed in a ratio of 1:1 with the matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid), and 1.5 μl of the mixture was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro (Applied Biosystems) MALDI-TOF mass spectrometer. In order to increase the sensitivity of the spectrometer, external calibration was applied with six different peptides (Protein MALDI-MS calibration kit; Sigma).

Recombinant DNA techniques. Standard genetic manipulations were performed essentially as described by Sambrook et al. (23). Plasmid pIL3BTC, encoding the nisin modification machinery (19), and plasmid pNZnisA-E3 (8) were received as a kind gift from BioMaDe Foundation, Groningen. In order to construct mutants of nisin, plasmid pNZnisA-E3 was mutagenized using the round PCR method with 5′-end-phosphorylated primers as described earlier (19). Plasmid isolation was performed by means of the plasmid DNA isolation kit

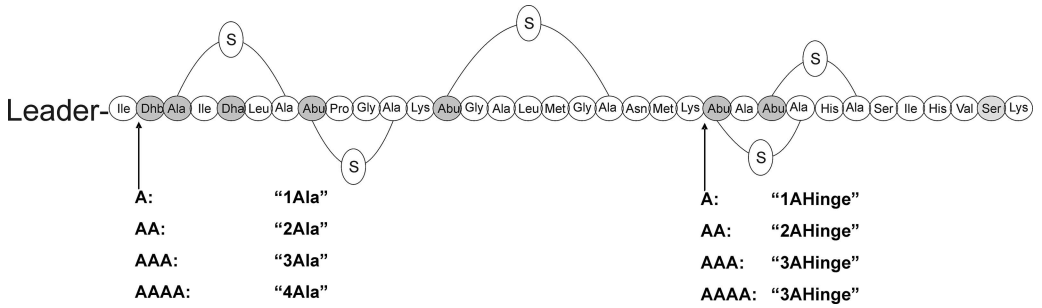

FIG. 1. Schematic representation of the primary structure of prenisin A and nisin mutants which were examined in the study. Leader, amino acid sequence MSTKDFNLDLVSVSKKDSGASPR.

(Roche Applied Science). Restriction analysis was performed with restriction enzymes from Fermentas. DNA ligation was performed with T4 DNA ligase (Fermentas), and round PCR amplification was done with Phusion DNA polymerase (Finnzymes).

Protein expression and purification. Supernatant samples containing antimicrobial peptides were prepared essentially as described before (19). Briefly, *L. lactis* NZ9000 containing plasmids pIL3BTC and pNZnisA-E3 or its derivative was grown overnight in GM17 medium supplemented with appropriate antibiotics. The preculture was diluted to 1:50 in minimal medium containing appropriate antibiotics and 0.5 ng/ml of nisin as an inducer. Cells were grown for an additional 24 h and were collected by centrifugation. The resulting supernatant was filtered through a 0.2-μm filter and subjected to fast protein liquid chromatography (FPLC). Supernatant was mixed 1:1 with a wash buffer of 50 mM lactic acid and applied to a HiTrap SP Sepharose (GE Healthcare) column for cation-exchange chromatography. Bound peptides were washed with 50 mM lactic acid and eluted with 50 mM lactic acid and 1 M NaCl. FPLC was conducted with an Akta purifier (Amersham Biosciences).

Growth studies. The antimicrobial activities of FPLC-purified prenisin and its mutants were tested against a sensitive *L. lactis* strain. Since leader cleavage is needed to liberate active nisin, a nisin-sensitive indicator strain producing the NisP leader peptidase (NZ9000/pNGnisPT) was used. The MIC of an antimicrobial peptide was determined by growth in 96-well microtiter plates by using a similar method as described before (14). Briefly, overnight cultures of the indicator strain, NZ9000, which contains pNGnisPT, were diluted into fresh medium and grown to an optical density at 660 nm of 0.5. Subsequently, cells were diluted 10 times, and 150-μl aliquots were mixed with 50 μl GM17 medium supplemented with 5 μg/ml chloramphenicol; 0.5 ng/ml nisin, necessary for expression of NisP; and various concentrations of antimicrobial peptides. The growth of the cells was monitored at 650 nm every 10 min for 16 h by means of a multiscan photometer (Thermomax microplate reader; Molecular Devices). The MIC was determined as the minimal concentration of an antimicrobial peptide which caused growth inhibition (optical density at 650 nm of <0.2) at a time when the indicator strain grown in parallel (without any antimicrobial peptide) reached stationary phase.

Trypsin cleavage. FPLC-purified peptides were subjected to tryptic cleavage. Peptides were incubated in a buffer containing 100 mM Tris (pH 8.0), 100 mM NaCl, and 2 mM $CaCl_2$ with a freshly prepared trypsin solution of 50 μg/ml for 1 h. Subsequently digested peptides were analyzed by Tricin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and MALDI-TOF spectrometry.

RESULTS AND DISCUSSION

Is the topological "reading frame" of nisin important for dehydration reactions? The 23 N-terminal amino acids of the nisin prepeptide compose the so-called nisin leader sequence. This stretch of amino acids was postulated to bear several important functions. Its presence keeps the mature peptide inactive, and only the cleavage of the leader sequence liberates the antimicrobial activity of nisin (25, 26). Next, mutations of amino acids which comprise the leader peptide strongly influence the secretion and production of the nisin precursor, which indicates the importance of the leader peptide in the maturation process (26). Recently, in vitro activity of NisC was reconstituted, and the leader sequence of nisin was shown to fulfill a role in NisC recognition and/or binding (13). Residues involved in a putative binding cleft for the nisin leader were proposed based on the NisC X-ray structure (13).

Clearly, the nisin leader sequence is important for cross talk between nisin and its modification machinery. Collectively, all these findings suggest that the leader sequence of nisin is indispensable for modification reactions to occur. Most likely, one of the functions of the nisin leader sequence is the binding and positioning of the prepeptide for the modification enzymes.

Here, we ask whether the distance between modifiable serines and/or threonines and the leader peptide, as well as the topology of these amino acids within the prepeptide, plays a role in the nisin modification reactions. To study this question, alanine insertional site-directed mutagenesis was performed. One to four alanines were consecutively introduced behind either isoleucine at position 1 or similarly behind lysine 22 in the hinge region (Fig. 1). All mutants were successfully overexpressed to similar levels in *Lactococcus lactis* NZ9000 together with the modification and transport machinery coded for by the plasmid pIL3BTC.

Dehydration pattern of prenisin and its insertional mutants. In order to study the dehydration pattern of wild-type prenisin and the eight prenisin mutants, prepeptides from overnight culture supernatants were purified on C_{18} column material by use of the ZipTip process described above and subsequently analyzed by MALDI-TOF mass spectrometry. The analysis of wild-type prenisin showed four distinct mass peaks that correspond to theoretical masses of prenisin which contain either eight or seven dehydrated residues and possess either an N-terminal methionine residue or not (Fig. 2). In addition, a minor peak which corresponds to prenisin species with only six dehydrated residues was observed (Fig. 2). Table 2 presents the obtained mass values for all eight prenisin mutants, which all show dehydration patterns similar to that of wild-type prenisin. All eight mutants show eight and seven dehydrated residues which correspond to the predominantly obtained mass peaks and a minor peak that corresponds to six dehydrated residues, which, however, is found to be more abundant in mutants containing one or three Ala insertions (1Ala or 3Ala, respectively) (Fig. 1). The mass spectra of wild-type prenisin and its eight different mutants can be viewed in Fig. S1 to S9 in the supplemental material. In order to obtain

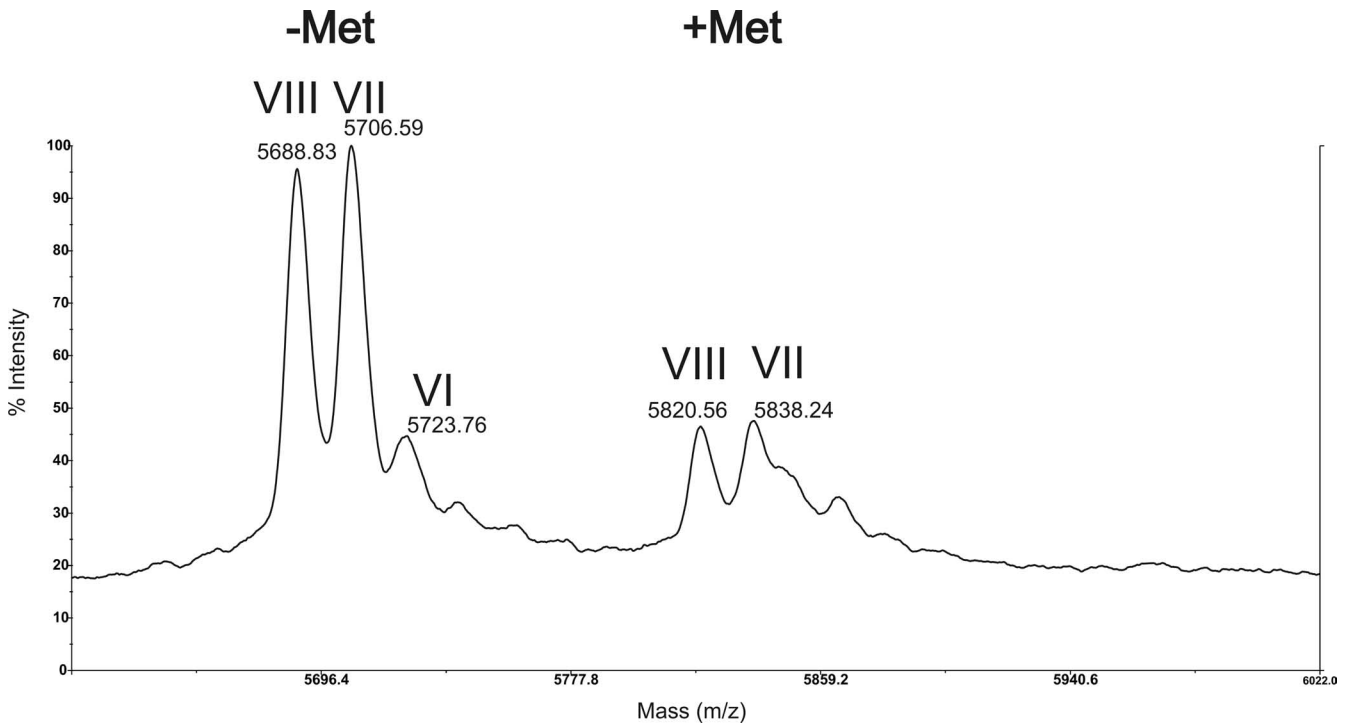

FIG. 2. Mass spectrum of prenisin produced by *L. lactis* NZ9000 which harbors pIL3BTC and pNZnisA-E3. Shown are four major peaks that correspond to the prenisin molecule which is dehydrated either eight (VIII) or seven (VII) times and either contains an initial methionine (+Met) or not (−Met). Additionally, a minor peak which corresponds to prenisin without initial methionine with six (VI) dehydrated residues is visible.

more subtle information on the dehydration extent of the mutants, they were purified by means of FPLC using a HiTrap cation-exchange column (Fig. 3). Subsequently, concentrated peptides were subjected to the ZipTip process and MALDI-TOF analysis. Concentrated and purified peptides showed dehydration patterns similar to those presented above (data not shown).

The similar dehydration patterns of wild-type prenisin and the eight alanine insertion mutants strongly indicate that the relative distance between the leader peptide and dehydratable residues is not a main determinant for the dehydration reaction to occur. It appears that the only strict requirement for NisB to process a peptide is the presence of the leader sequence. The distance between the leader and the dehydratable residues does not seem to play a major role. Interestingly, the topology of the Ser and Thr residues, with respect to their location in the amphipathic molecule, also does not determine their dehydration. According to Rink et al., the hydrophobicity of the immediate surroundings of serines and threonines can have an influence on the dehydration reaction (19, 21). Almost opposite rules defining substrate specificity were described in a recent publication about LctM, an enzyme that modifies lacticin 481. In that case, a hydrophilic environment of serines and threonines did not preclude their dehydration (1). Even though LctM and NisB can both catalyze dehydration reactions, they are substantially different, and they do not share homology at the amino acid level. Therefore, differences in their substrate specificities are not entirely surprising. However, it is to be expected that the molecular details of enzymatic catalysis of the dehydrating enzymes of both the LanB and LanM classes are similar.

Protection against tryptic cleavage behind lysine 22 by ring D and/or dehydrobutyrine 23. It was suggested before that the presence of (methyl)lanthionine ring structures and/or dehydrated residues in close proximity to lysine residues protect against trypsin cleavage (13). Here, we demonstrate that lysine 22 is protected against tryptic cleavage only when immediately followed by a (methyl)lanthionine and/or dehydrobutyrine. The insertion of one alanine between lysine 22 and ring D exposes the lysine cleavage site to the proteolytic action of trypsin, resulting in the disappearance of the nisin band on the gel (see the arrow in Fig. S10 in the supplemental material).

The N-terminal part of nisin is more sensitive to amino acid insertions than the hinge region of the molecule. We purified wild-type prenisin and eight mutants (Fig. 3). All these peptides still contain leader sequences which keep them in an inactive state. In order to analyze the effects of the introduced mutations on the antimicrobial activity of nisin, we used a strategy that has been reported before (8). We used a sensitive indicator strain, i.e., *L. lactis* NZ9000, which harbors plasmid pNGnisPT for the overproduction of the native nisin protease NisP. The indicator strain incubated with a purified prenisin species produces NisP. NisP cleaves off the leader peptide of prenisin, thus liberating antimicrobial activity, which inhibits the growth of the indicator strain. Growth experiments were performed in a 96-well-plate format by a series of dilutions as described previously (14), and the growth was monitored for 16 hours. Purified prenisin species were compared with nisin pu-

TABLE 2. Dehydration of prenisin and its mutants by NisB, analyzed by MALDI-TOF mass spectrometry

Prenisin peptide	No. of dehydrated residues	Mass (M + H+) without Met1 (Da)	
		Observed	Calculated
Wild type	8	5,688.83	5,688
	7	5,706.59	5,706
	6	5,723.76	5,724
1Ala	8	5,759.41	5,759
	7	5,777.39	5,777
	6	5,795.31	5,795
2Ala	8	5,832	5,830
	7	5,849.82	5,848
	6	5,866.98	5,866
3Ala	8	5,900.96	5,901
	7	5,918.92	5,919
	6	5,936.97	5,937
4Ala	8	5,971.74	5,972
	7	5,989.55	5,990
	6	6,006.96	6,008
1A hinge	8	5,759.38	5,759
	7	5,777.09	5,777
	6	5,794.73	5,795
2A hinge	8	5,831.74	5,830
	7	5,849.55	5,848
	6	5,866.39	5,866
3A hinge	8	5,904	5,901
	7	5,921.38	5,919
	6	5,939.19	5,937
4A hinge	8	5,974.83	5,972
	7	5,992.37	5,990
	6		6,008

rified by high-pressure liquid chromatography. Table 3 shows the antimicrobial activities of wild-type nisin and eight mutants.

In the case of insertional changes in the N-terminal part of nisin, no activity was liberated by NisP (Table 3). The insertion of only one alanine behind the N-terminal isoleucine increases the MIC of this mutant above 1,280 ng/ml. The insertion of alanine in the N-terminal part of the nisin molecule could affect the processing of prenisin by NisP, which was used in the experiment presented in Table 3. Therefore, we performed additional experiments using trypsin instead of NisP to process these mutants. N-terminal insertional mutants were properly cleaved by trypsin, as checked by Tricine-polyacrylamide gel

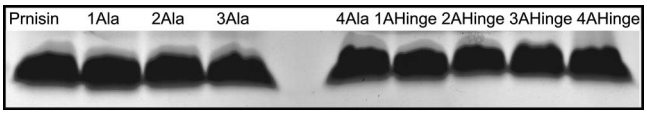

FIG. 3. Prenisin and its eight mutants were purified by FPLC and concentrated as described in Materials and Methods. These peptides were visualized by means of Tricine-polyacrylamide gel electrophoresis.

TABLE 3. Biological activities of nisin and mutant prenisin species

Antimicrobial	MIC (ng/ml) for *L. lactis* NZ9000/pNGNisPT[a]
Nisin	20
Prenisin WT[b]	20
Prenisin-1Ala	>1,280
Prenisin-2Ala	>1,280
Prenisin-3Ala	>1,280
Prenisin-4Ala	>1,280
Prenisin-1A hinge	40
Prenisin-2A hinge	320
Prenisin-3A hinge	640
Prenisin-4A hinge	640

[a] Indicator strain.
[b] WT, wild type.

electrophoresis and MALDI-TOF analysis. The activity of the cleaved mutants was assayed against NZ9000 indicator strains. The mutants did not show any detectable antimicrobial activity, suggesting that the lack of activity is related to alanine insertions and not to the lack of NisP processing (data not shown). The data indicate that the N-terminal part of nisin is very sensitive to insertional changes, which most likely relates to the interference of the introduced amino acids with the formation of the "glove-like structure" responsible for nisin binding to lipid II (4). However, the possibilities that these alanine additions influence cyclization reactions performed by NisC and that the observed lack of activity is due to incorrect ring structure formation in mutants cannot be excluded.

Similar Ala additions in the hinge region have less-severe effects on nisin antimicrobial activity than Ala additions in the N-terminal region. The activities of the hinge mutants prove that they are cleaved by NisP and that at least rings A, B, and C are closed. In the case of the 1A hinge, it is most likely that rings D and E are also closed. The insertions of one to four alanines in the hinge region have a weaker effect on antimicrobial activity of the molecule than do insertions in the N-terminal region. This probably relates to the fact that this region does not directly interfere with the binding of nisin to its docking molecule. However, the alanine additions elongate the nisin molecule and can possibly influence pore formation and the spanning of the membrane by nisin. This may likely be the cause of the gradual decrease of antimicrobial activity of nisin mutants extended by the addition of one to four alanines. Alternatively, this extension may raise the antimicrobial effect against target organisms with a thicker lipid bilayer.

Dehydratable serines and threonines, as well as (methyl)lanthionines, are important in a variety of activities of lantibiotics. They were demonstrated to be important for antimicrobial activity, modulation of autolytic enzymes, and proteolytic stability of lantibiotics. Recent studies showed the relaxed specificity of nisin-modifying enzymes (6, 8, 9, 18–21), which opens up ways to use them as biotechnological tools to introduce thioether structures into a variety of valuable peptides. In order to achieve this goal, detailed knowledge of the substrate specificity of nisin-biosynthetic enzymes is required. It was demonstrated that NisB does not restrict its activity to the nisin template and can dehydrate a wide variety of peptides (6). Although NisB has been shown to display rather promiscuous substrate specificities, not all serines and threonines were

equally well dehydrated. In silico analysis of 37 primary structures of lantibiotics could not find a specific sequence motif which would encompass a modification signal (19). Instead, analysis of various lantibiotic amino acid sequences led to the conclusion that the hydrophobicity of neighboring residues of serine or threonine may influence its dehydration (19). Although a number of non-nisin peptides were successfully dehydrated by NisB (6, 19), dehydratable serines and threonines in these peptides occupied positions with respect to the nisin leader sequence that were identical or very similar to those in wild-type nisin. Here, we have examined in a systematic way whether the positioning of a dehydratable residue relative to the leader may influence their dehydration. Moreover, the topology of Ser and Thr residues in the amphipathic molecule changed considerably due to the inserted Ala residues. Analysis of the dehydration pattern of the eight mutants, in which the distance between serines and threonines and the leader sequence was changed by one to four alanine residues, indicates that the positioning relative to the leader sequence does not play a crucial role for a dehydration reaction to occur. Moreover, we have analyzed the antimicrobial activities of the alanine insertion mutants and noticed that mutational changes of the N-terminal part of nisin, which is responsible for high-affinity lipid II interactions, are less well tolerated than a similar mutation in the hinge region of the molecule.

The broad substrate specificity of the nisin dehydratase holds promise for its use as a tool to introduce dehydrated amino acids into a wide variety of biotechnologically valuable peptides in order to stabilize these peptides and modulate their activity. The shifting of lanthionine ring positions may be combined with the randomization of rings A and B, as shown previously (18), thus further enlarging the presently known tremendous possibilities to engineer nisin variants with desired characteristics.

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